the University of Alabama in Birmingham / UNIVERSITY STATION / BIRMINGHAM, ALABAMA 35294
the Medical Center / DEPARTMENT OF MICROBIOLOGY

August 6, 1974

M E M O R A N D U M

TO: Paul Berg, David Baltimore, Herbert W. Boyer, Stanley N. Cohen, Ronald W. Davis, David S. Hogness, Daniel Nathans, Richard Roblin, James D. Watson, Sherman Weissman and Norton D. Zinder

FROM: Roy Curtiss III

SUBJECT: Potential Biohazards of Recombinant DNA Molecules

Introduction.

I heartily endorse the aims, but not necessarily the scope, of your recommendations contained in your letter as recently published in Science [185:303 (1974)] and Nature [250:175 (1974)]. I personally pledge to cease Type I experiments (to construct bacterial plasmids that are not now known to exist) that I was currently engaged in and not to initiate Type 2 experiments (to construct viral-viral or viral-plasmid recombinant molecules) which are not part of my current research plans.

I shall take this opportunity, however, to argue for a broadening of the definitions of both Type 1 experiments (to include construction of all hybrid bacterial plasmids regardless of the known or unknown phenotypic traits specified) and Type 2 experiments (to include construction of all viral-viral and viral-plasmid hybrids regardless of whether the viruses come from bacteria, plants or animals and whether the plasmids come from prokaryotic or eukaryotic organisms) and strongly urge a temporary ban on Type 3 experiments (to construct recombinant molecules between eukaryotic DNA and either bacteriophage or bacterial plasmid DNA). I also suggest a definition for a Type 4 experiment as one involving the introdution of either viral, extrachromosomal or chromosomal DNA, whether naturally occurring or recombinant, from one species to another that is not a normal host for such DNA if it is possible that such introduction might result in a new pathogenic organism or agent or diseased state capable of transmission from one generation to the next, to voluntarily cease such experiments and to argue for a similar cessation of such experiments until potential biohazards can be assessed and means to cope with them established.

In order to justify my points of view on these matters, I will discuss (a) the nature of the experiments we attempted or contemplated and the potential biohazards associated with them; (b) the great diversity of functional attributes specified by bacterial plasmids; (c) some potential origins for plasmid genes and replicons in nature; (d) the pathogenicity of Escherichia coli and the contribution of plasmids to its increasing virulence; (e) the consequences of the ubiquitious conjugal transfer of genetic information to and from the sexually adept E. coli; (f) possible areas of genetic engineering research in the future and some further biohazards associated with this research; (g) my definitions of the types of experiments to be considered for temporary cessation and the reasons for my suggested modifications of your original recommendations; and (h) a general treatment of suggested means for dealing with the biohazards associated with this research in the belief that these biohazards can and should be controlled so that this important area of investigative research can continue for the betterment of all.

Plasmid Research on Streptococcus mutans.

Streptococcus mutans is the principal etiological agent of dental caries, and as such is probably one of the most costly infections of humans. Recent work has demonstrated that cariogenicity of S. mutans is due to the production of an isoluble dextran-like polysaccharide that permits adherence to the tooth surface, that loss of the ability to produce this polysaccharide results in avirulence and that the production of this polysaccharide material is plasmid specified or controlled. In order to determine unequivocally whether the S. mutans plasmids directly specify the synthesis of one or more of the glucosyltransferases involved in the production of dextran-like polysaccharide from sucrose, we recently attempted to transform a restriction-less minicell-producing strain of E. coli with such plasmid DNA. As might be expected, our attempts at a Type 4 experiment to isolate E. coli minicell-producing transformants that could utilize sucrose as an energy source because of the presence of S. mutans plasmid-specified glucosyltransferases did not meet with success because such plasmid DNA could not replicate and/or express its genetic information in E. coli and/or did not contain the structural genes for glucosyltransferase(s). The next logical step to elucidate the gene products of the S. mutans plasmid and thus determine the mechanisms for virulence of this microbe and the means to curtail it, would be to construct a hybrid between this plasmid DNA and a plasmid that normally inhabits E. coli by use of the EcoRI endonuclease (a Type 1 experiment by my redefinition but not literally excluded by the definition given in your letter to Science and Nature). While the goals of such research are seemingly beneficial, the potential of endowing E. coli with the ability to occupy a new ecological niche and indeed to become cariogenic is somewhat frightening. In reality, however, the greatest fear that I perceived prior to abandoning this line of thought was the fact that such a microbe might also produce other streptococcal proteins which might, upon repeated exposure to humans, induce myocarditis and/or glomerulonephritis - two frequent complications associated with streptococcal infections.

Is this plausible? Why should carrying this plasmid ... to occupy ... Maybe ... but ... to ... environment?

What means necessity for ... through dissemination of ... plasmid.

In order to be complete, I should also mention some additional types of genetic engineering experiments that we considered as potential means to displace virulent S. mutans from its normal ecological niche and thus to reduce dental caries. One line of attack would be to couple a bacterial gene coding for dextranase to a plasmid present in a "normal harmless" bacterial inhabitant of the oral cavity. A strain of bacteria that produced copious quantities of extracellular dextranase could then be selected, introduced into the oral cavity and there act to enzymatically destroy the dextran-like polysaccharide and thus inhibit and/or prevent colonization of the tooth surfaces by virulent S. mutans. A second, more far out approach would be to attempt to couple eukaryotic DNA sequences coding for anti-S. mutans immunoglobulins to plasmid DNA, to introduce this hybrid plasmid into our "normal harmless" inhabitant of the oral cavity, and then use this bacterium to combat S. mutans in situ. I should hasten to add that I am well aware that the use of such "microbiological warfare agents" would initially upset the ecological balance to the detriment of S. mutans but that the inherent genetic instability of DNA and the imposed selection pressures would eventually result in a new ecological balance that could even result in abnormally high oral titers of variant, yet virulent, S. mutans. Nevertheless, the eventual experimental testing of such possibilities under very carefully controlled circumstances seems warrented in view of the lack of existing effective and theoretically possible means to curb infection by this pathogen.

Functional Attributes of Plasmids.

The information contained in this and the following three sections which concern the role of plasmids in the pathogenicity, sexuality and evolutionarily significant adaptability of E. coli and other microbes will serve as major additional reasons for my suggested modifications of your definitions and recommendations as published in Nature and Science.

Bacterial plasmids confer a far greater diversity of phenotypic traits on the bacteria that possess them than the rather restricted instances of drug resistance and toxin production as used in your narrow definition of plasmids not to be used in Type 1 experiments. There are (a) conjugative plasmids that confer few if any phenotypic traits other than the capability of a bacterium that possesses it to conjugally donate itself, other non-conjugative plasmids and/or chromosomal DNA to a suitable recipient bacterium. (i.e., F of E. coli; FP2, FP5, FP39, pfdm and K of Pseudomonas; P of Vibrio cholera; and SCP1 of Streptomyces). (b) R plasmids, both conjugative and non-conjugative, that frequently confer multiple resistance to antibiotics and, in addition, resistance to mercury, cadmium, nickel, cobalt, zinc and/or arsenicals and more rarely are associated with additional phenotypic traits such as ability to ferment lactose or raffinose or produce H_2S. The latter plasmids are undoubtedly the consequence of plasmid-plasmid recombination in nature. (Reports of various types of naturally occurring plasmid recombinants appear with increasing frequency each year.)

R plasmids naturally occur in or can be conjugally transferred to members of twenty-six bacterial genera. (c) Plasmids that specify the production of antibiotics such as the bacteriocins of many gram-negative microorganisms and Clostridium as well as the "true" antibiotics produced by Streptomyces. (d) Plasmids then confer on their hosts new metabolic properties, i. some of which are uncommon in such species and thus contribute to difficulty in establishing an accurate clinical identification (i.e., the lac^+ plasmids of Salmonella, Proteus, Klebsiella and Erwinia: the sucrose-utilization plasmid of Proteus; the H_2S-production plasmids of E. coli; the urease-production plasmids of Proteus and Streptococcus), ii. some of which enable the bacterium to establish in new ecological niches and/or utilize unique energy sources (i.e., the nitrogen fixing plasmid of E. coli; the octane-, camphor-, mandelate-, napthalene- and salicylate-utilization plasmids of Pseudomonas) and iii. some of which just alter physiological functions (i.e., sporulation in Bacillus, pigment production in Erwinia and Streptomyces, amino acid excretion in E. coli). (e) Plasmids that contribute to bacterial virulence such as the plasmids that specify production of enterotoxins in E. coli and Streptococcus, K surface antigens in E. coli that facilitate adherence to the intestinal mucosa, endotoxins in E. coli that thwart phagocytosis, factors that facilitate penetration of the intestinal mucosa in E. coli; urease in Proteus and E. coli that facilitates infection of the urinary tract, and the insoluble dextran-like polysaccharide of S. mutans that is essential for colonization and virulence. There is also reason to believe that virulence in Agrobacterium tumfaciens to cause crown gall tumors is plasmid specified. (f) Plasmids that affect mutability or have suppressor activity as described in E. coli and Salmonella. (g) Plasmids that are phage or defective phage genomes such as P1, P1CM, λdv, λNN, φamp, etc., and quite possibly some of the prophages whose site of replication in the bacterium is unknown and which may be responsible for phage conversion endowing bacteria with the ability to produce exotoxins as in Corynebacterium and Clostridium, somatic antigen (endotoxin) production in Salmonella, fibrinolysin production in Staphlococcus, erythrogenic toxin production in Streptococcus, etc. (h) Cryptic or silent plasmids which confer on their hosts no known phenotypic trait, are of a great diversity of sizes but which have been detected in members of sixteen bacterial genera.

In summary, plasmids with one or more of the above-described properties have been found in or transferred to members of thirty-three different genera [i.e., Achromobacter, Aerobacter, Aeromonas, Agrobacterium, Aklalescens, Arthrobacter, Bacteriodes, Bacterium, Bacillus, Bartonella, Chromobacterium, Citrobacter, Clostridium, Enterobacter, Erwinia, Escherichia, Hafnia, Klebsiella, Micrococcus, Neisseria, Paracolobactrum, Proteus, Providencia, Pseudomonas, Rhizobium, Salmonella, Serratia, Shigella, Staphlococcus, Streptococcus, Streptomyces, Vibrio and Yersinia (formerly Pasteurella)]. In addition, the occurrence of conjugal chromosome transfer in Mycobacterium, Micromonospora and Nocardia and presumptive evidence in Caulobacter might suggest the presence of plasmids in members of these genera as well.

Potential Origins for Plasmid Genes and Replicons.

Although a comprehensive consideration of the origin of plasmid genes and replicons would be highly speculative and would confront enumerable chicken-egg paradoxes, there is sufficient data on several points to provide specific mechanisms and/or facts that are relevant to the problem of formation of recombinant plasmids. A ubiquitous trait of prokaryotic organisms that harbor or produce potentially lethal or harmful agents or substances is their immunity to the action of such agents or substances. This is also true for the antibiotic-producing Streptomyces. In the latter case it has been observed that various species make enzymes that specifically inactivate the respective antibiotic produced and this led to the suggestion that these drug-inactivating enzymes might be involved in the biosynthesis of antibiotics so as to render the Streptomyces strains immune to their action. It follows from this that the Streptomyces most likely also make enzymes that reactivate the antibiotic during its transport out of the cell. The startling recent discovery is that the drug-inactivating enzymes from Streptomyces and those specific by R plasmid-containing gram-negative bacteria have the same substrate specificity, mode of action, etc. and this has led to the suggestion that the R plasmid genes coding for drug-inactivating enzymes originated in the antibiotic-producing Streptomyces. The time in evolutionary history and the mechanism for this type of presummed gene flow are as yet unknown. Nevertheless, Streptomyces have plasmids that can now be isolated as covalently closed circular DNA molecules and some of these plasmids seem to code for the biosynthesis of antibiotics. It also follows that genes probably already exist in nature to specify the synthesis of enzymes to inactivate all antibiotics that are biologically produced - at least by Streptomyces.

Origins for genes that permit the replication of plasmids as replicons are undoubtedly diverse in view of the large numbers of different plasmid incompatibility groups. It has been known for some time that genetic inhomology between donor and recipient DNA often leads to unstable partially diploid recombinants. This is certainly true with regard to the recombinants arising from intergeneric matings in whcih chromosomal DNA is conjugally transferred between Escherichia, Salmonella, Shigella, Proteus, and Klebsiella strains. Upon close examination of these partially diploid recombinants, it has been found that they possess covalently closed circular DNA molecules whose molecular weight is proportional to the genetic content of the extrachromosomal fragments. Thus intergeneric conjugal chromosome transfer provides a means to generate autonomously replicating plasmids. I speculate now by proposing that the genes that permit this replicative activity originate from defective prophage genomes present in the DNA fragment from the donor parent. The reasonableness of this phage origin for essential replicon genes is further supported by the facts that temperate phages such as λ of E. coli and pf16 of Pseudomonas putida can be altered to become the autonomously replicating λdv and λsusN plasmids and the conjugative pfdm plasmid, respectively.

Virulence of E. coli as a Pathogen.

E. coli is often thought of as a harmless inhabitant of the intestinal tracts of animals. I would like to take this opportunity to indicate that E. coli is a pathogen with strains exhibiting various degrees of virulence. Indeed, infections with enteropathogenic strains of E. coli are probably responsible for the vast majority of diarrheal diseases and other enteric disorders among children and adults in the U.S.A. Furthermore, E. coli is one of the three main killers associated with patients dying of septacemias that secondarily arise because of diseases or states such as cancer, immune deficiency, transplantation, surgery, ulcers, appendicitis, peritonitis, etc. Consequently, infections due to virulent strains of E. coli result in significant economic losses in terms of diagnosis and treatment expenses and costs associated with morbidity and mortality and thus constitute one of our major medical problems.

E. coli's status as a medically important pathogen is only now beginning to be realized and this is due in part to the fact that E. coli infections were rarely encountered ten to twenty years ago except in cases of infantile diarrhea. Among E. coli strains isolated twenty years ago, plasmids were rather rare and when found usually were of but one kind. Today thirty to fifty percent of all E. coli strains harbor plasmids and among E. coli strains from hospital patients and personnel, ninety percent of more harbor plasmids with a mean number of molecularly distinct plasmids being about three per strain. Admittedly the methodology of detecting plasmids has markedly been improved during this same interval of time, but I consider this increase to be very real and to represent a major evolutionarily significant change in the genetic potential of E. coli and of other microorganisms as well.

In terms of plasmids and/or genetic information that could contribute to the virulence of E. coli, it is feasible to endow an ordinary E. coli (a) with a prophage and a Col plasmid to facilitate displacing normal flora and thus contributing to colonization; (b) with a plasmid that specifies a K surface antigen that facilitates adherence to the intestinal mucosa; (c) with an Ent plasmid that elicits the production of enterotoxins whose mode of action is similar to, but less severe, than that of the cholera enterotoxin; (d) with either a plasmid or chromosomal gene specifying a potent endotoxin to reduce the host's ability to phagocytize the invading microbe; (e) with either a plasmid or a chromosomal gene that permits penetration of the intestinal mucosa similar to the penetrating ability of Shigella strains; (f) with a urease-specifying plasmid to facilitate urinary tract infections; (g) with plasmids such as those allowing the production of H_2S, etc. which are not normal properties of E. coli to make clinical diagnosis difficult; and lastly (h) with a multiple drug-resistance R plasmid so that the infection caused by this multiple plasmid-containing E. coli is untreatable. Clearly, that would be one hell of a virulent infectious agent. Although such a microbe could readily be constructed in the laboratory it can also arise in nature. Indeed enteropathogenic E. coli strains with several combinations of the above mentioned attributes have recently been isolated and characterized.

Sexuality of Bacteria and E. coli in Particular.

The substantial increase in bacteria with plasmid-specified information is undoubtedly a consequence of environmental changes that provide selection pressures (i.e., the use of antibiotics as feed additives for cattle, fish and poultry, etc.) and increased opportunities for conjugal plasmid and/or chromosome transfer (i.e., due in part to increased levels of water pollution, etc.). A great deal is known about the genetic, physiological and molecular details of conjugal genetic transfer in E. coli and I personally consider that E. coli's capabilities in these activities to probably be unequaled by any other living organism (even bedbugs). E. coli, which normally divides every 20 to 30 minutes, can sustain the conjugal act for up to two to three hours, and most likely does so in the absence of vegetative chromosome replication and cell division. Mating partners are randomly selected without regard to size or shape and multiple matings between a cell of one mating type and several of the other are a frequent occurrence in laboratory experiments at least. During conjugation, the donor genetic information is simultaneously replaced during its transfer to the recipient such that a donor cell is immediately able to engage in a second conjugal act following cessation of the first. The transfer of a conjugative plasmid from a donor to a recipient results in the recipient being converted to a donor such that two donor cells now exist. This poses a paradoxical situation since this would suggest that all E. coli strains should eventually be converted to donors and, since it has been known for a long time that matings between cells harboring the same plasmid are rare due to entry exclusion, would act as a barrier to conjugal gene flow. Such a situation only reduces gene flow about 100-fold, however, since homosexual matings in bacteria often lead to progeny containing recombinant plasmids and/or chromosomes unlike the infertility associated with homosexual couplings in other organisms. Furthermore, there are in excess of twenty groups of conjugative plasmids present in E. coli and other gram-negative bacteria as defined by incompatibility, donor pilus type and entry exclusion phenotypes and cells possessing a conjugative plasmid of one group mate very well with cells possessing a conjugative plasmid from any other group. Thus E. coli and other gram-negative microbes have solved this problem of the ubiquity of plasmids by becoming bisexual in that a given cell can and does act as both a competent donor and fertile recipient during the same conjugal act.

E. coli also occassionally chooses its mating partners without regard to their taxonomic designations and is known to donate and/or receive plasmid and/or chromosomal information from members of over twenty genera. The demonstrated occurrences of these types of intergeneric gene flow are somewhat rare but the laboratory methods currently used for their detection are rather insensitive in that restriction-less recipients from a number of genera are seldom, if ever, used. The confines of laboratory measurement (i.e., one in 10^{10}) are not present in nature, however, where the total microbial populations in soil, polluted water and in and on various eukaryotic organisms are such as to make events whose probability of occurrence is the reciprocal of Avagodro's number

not impossible. Indeed, conjugal gene transfer has been demonstrated to occur in soil, the nodules on the roots of leguminous plants and in the intestinal tracts of fish, poultry, rodents, cattle and humans. Plasmid and chromosome transfer also occur with equal frequency under both anaerobic and aerobic environments. Thus a great diversity of environments, some of which are "improving" because of greater pollution and/or microbial acquisition of plasmids that expand the ecological niches that they occupy, are suitable for conjugal gene flow.

The process of bacterial conjugation may even have been adapted for other uses. Virulent Agrobacterium tumefaciens that cause crown gall tumors in plants possess plasmids whereas avirulent strains do not. Thus plasmids may be responsible for virulence in this microbe. The observation that tumor induction is dependent on the presence of intact, viable, metabolically active A. tumefaciens cells and susceptible plant tissue leads me to suggest that infectivity leading to tumor formation may involve a conjugation-like act between the respective prokaryotic and eukaryotic cells. If so, then the fact that E. coli and other gram-negative bacteria can conjugally transmit plasmids to A. tumerfaciens leads to interesting ramifications. In this regard, it is fairly well established that phage, plasmid and bacterial chromosomal DNA can be taken up, survive and expressed in plants.

Genetic Engineering Research in the Future.

One could reasonably ask whether the research recommended for temporary cessation until biohazards are enumerated and methods for their control established should even be done? I personally believe that many of the experiments should be done. I am thus an advocate for and not an opponent of genetic engineering research. Additional information on the transcriptional and translational control of prokaryotic genetic information in eukaryotes and vice versa is of fundamental importance. If fidelity is achieved in the latter case, we could achieve a better understanding of genetic regulation of eukaryotic DNA as it relates for example to differentiation of the immune system and aberrances associated with cancer and hereditary genetic defects as well as to be able to use microbial systems for the production of useful products such as insulin, growth hormone, etc., and even useful foodstuffs such as vitamins, casein (and thus eliminate the need for cows), etc. Inadvertant release of such microbes that produced immunoglobulins, blood group or histomcompatibility antigens, and a multitude of other protein types could, however, interfere with success of blood transfusions and organ transplantations and induce states of hypersensitivity. The release of microbes that produced hormones might have even more pronounced deleterious effects.

The use of recombinant DNA molecules composed of virus, plasmid and/or chromosomal genetic information to endow plant species with, for example, improved ability at photosynthetic CO_2 fixation; capability to fix nitrogen,

increased quantity or improved quality of protein, etc. would contribute to the goals of the green revolution and possibly reduce the need for chemical fertilizers and the associated pollution, eutrophication, etc. that their use entails. The genetic manipulation of the marine algae and blue-green bacteria that are responsible for production of about ninety percent of the earth's oxygen supply to better resist and/or utilize the increasing levels of pollutants in the seas also merits consideration.

Genetic engineering of microorganisms is also highly relevant in that it is conceivable to create E. coli derivatives that utilize hydrogen for the production of energy, that can carry out photosynthesis, and that can fix nitrogen. The development of microbes to produce compounds useful in industry, agriculture and medicine also has merit. Even the creation of potential new pathogenic strains could be of great importance. In the United States, typhoid, cholera and plague are not problems because of sanitary engineering, public health measures and good personal hygiene. These factors may be insufficient, however, to preclude epidemics in the future. Current sewage treatment procedures when used by a city of ten million population result in a level of water pollution when treated wastes are discharged into the nearest body of water that are equivalent to the discharge of the untreated wastes from a city of 100,000 to one million population. Thus water pollution is becoming more severe and water quality in declining. In addition, R plasmids conferring resistance to the drugs of choice in treating typhoid already exist in Salmonella typhosa and can readily be transmitted to Yersinia pestis, the causitive organism of plague. Clearly the development of safe, effective vaccines is therefore of prime importance and the use of hybrid bacterial strains for this purpose deserves special attention.

One can also question whether E. coli should be used for these types of research. Again I must answer this in the affirmative even though I have some trepidation over this. At least a billion dollars has been spent by the countries of the world for research on this organism and without doubt we know more about E. coli than any other living organism. To not utilize this knowlege would be wasteful. Furthermore, the selection of some other microbe for these studies would be most difficult and potentially hazardous. For example, the absence of a conjugal transfer system in the chosen bacterial species might solely be due to the fact that insufficient effort by scientists trained and interested in such discoveries had been made. If the selected microbe was a gram-negative microorganism then I think it is likely that conjugation with a member of any one of the more than twenty gram-negative genera that are known to carry out conjugal gene transfer could and would occur. The use of a gram-positive microorganism also offers no advantages since conjugal gene transfer is now known to occur in some groups and gene transfer by transduction and/or transformation in others. The use of gram-positive spore formers seems extremely hazardous and I see no advantages in using soil microorganisms or potential plant pathogens. In all instances severe, even though different, biohazards exist and there would be no lessening of the need for effective means

to cope with these biohazards. Thus I believe E. coli to be the most suitable microorganism for these studies. Obviously, however, this matter requires further thought and debate.

Suggested Modifications of Original Proposals as Published in Science and Nature.

As a preface to the comments that follow, let me indicate that your definitions of Types 1 and 2 experiments contain several bothersome omissions; that your recommendations and cautionary commentary are based, for the most part, on a concern for human welfare and seem to ignore the fact that plants are an important indispensable component of the entire ecosystem; and that there are severe potential biohazards associated with many types of experiments that are not covered by your restricted definitions of Types 1 and 2 experiments or for which only caution is recommended. I therefore propose modifications of your definitions and recommendations to take into account these considerations as well as the facts and arguments presented in my foregoing commentary and in your letter to Science and Nature.

New Recommendations and Definitions

I have suggested definitions below for four classes of experiments. Since I find it extremely difficult to draw a sharp line of demarcation between those experiments that are associated with significant potential (or real) biohazards and those that are not, I have chosen to draft these definitions so that they are all inclusive and will therefore be invariant with time and unaltered by new discoveries. My recommendations pertaining to the temporary cessation of such experiments, however, are predicated on the realization that there is a gradation of severity of potential biohazards for experiments of a given type and that a decision on the performance of any given experiment will have to be made with the particulars of that experiment in mind. It should therefore be the obligation of each individual scientist, in consultation with colleagues, to evaluate the existance and severity of biohazards and to then decide whether the experiment should proceed or be temporarily deferred until means to contend with the biohazards are established and implemented. My rationale for this approach to the problem is based on a desire to see that important areas of genetic research continue when biohazards are minimal or nonexistant.

Type 1 Experiments: Construction by biochemical or genetic techniques of all, new, autonomously replicating bacterial plasmids that specify combinations of phenotypic traits, whether known or unknown, and the introduction of such recombinant plasmids into either prokaryotic or eukaryotic organisms.

Clearly the formation of a hybrid between the S. mutans plasmid and an E. coli plasmid constitutes a hazardous experiment in view of the potential to create a new pathogen. Similarly the construction of a hybrid between the conjugative R plasmids of S. faecalis and E. coli might potentiate conjugal transfer between gram-positive and gram-negative bacteria which would be a most unfortunate result. (A determination of the relatedness of these R plasmid

types is certainly important to know but should be investigated by DNA-DNA hybridization techniques.) Experiments such as these should therefore be deferred until the means to contend with the respective biohazards are established and implemented. On the other hand, the numerous plasmids in gram-negative bacteria are freely transmitted between species and genera and naturally occurring recombinant plasmids are rather frequent. Although hybrid plasmids combining new traits not now in existance should not be created, it would seem that much research in this area could continue if acceptable microbiological procedures are utilized.

Type 2 Experiments: Construction by biochemical or genetic techniques of all viral-viral and viral-plasmid hybrids regardless of whether the viruses come from bacteria, plants or animals and whether the plasmids come from prokaryotic or eukaryotic organisms and the introduction of such recombinant molecules into either prokaryotic or eukaryotic organisms.

Most of the experiments of this type should be temporarily deferred with the exception that construction of viral-viral hybrids involving two viruses that had the same host range and possessed other genetic, morphological and/or serological properties in common (i.e., λ and ϕ80) would be reasonably free from biohazards.

Type 3 Experiments: Construction by biochemical or genetic techniques of all hybrid molecules between chromosomal DNA from either prokaryotic and eukaryotic organisms and viral and/or plasmid DNA from either prokaryotic or eukaryotic organisms and the introduction of such hybrid molecules into either prokaryotic or eukaryotic organisms.

Except for the formation and use of specialized transducing phages, attempts to discover plant or animal transducing viruses by use of cell culture systems, and the use of specialized transducing phages or their DNA for studies of transformation and gene expression in eukaryotic cells in culture, other experiments of this type should not be performed at present.

Type 4 Experiments: Introduction of viral, plasmid, extrachromosomal or chromosomal genetic information from one prokaryotic or eukaryotic organism into another prokaryotic or eukaryotic organism that either does not now contain or is not now a normal host for such viral, plasmid, extrachromosomal or chromosomal genetic information if it is possible that such introduction might result in a new pathogenic organism or agent or diseased state capable of transmission from one generation to the next.

The definition of a Type 4 experiment is more narrowly conceived than those for the other experiments. Because of this I cannot think of an experiment of this type which is free from biohazards. Thus these types of experiments should be deferred.

Some of the reasons for these all-encompassing recommendations (which combine and would replace the first and second recommendations in your letter) are contained in your own letter and need not be reiterated here since I fully subscribe to them. Additionally, I am concerned with the biohazards associated with the creation and unintentional release of new pathogens, whether viral or bacterial, and organisms that contain new genetic information that could be transmitted to other prokaryotic or eukaryotic organisms and thus either directly or indirectly (as a consequence of mutation, recombination and seletion) inadvertantly alter the ecological balance between living organisms, the environment in which we live and/or interfere with successful practices in agriculture and medicine to control or treat diseases or disease states of plants and of animals including humans. The pathogenicity and sexual capabilities of E. coli (which is the organism most likely to be used in many genetic engineering experiments) serve as principle bases for my concern.

I agree with your third recommendation for the establishment of an advisory committee by the director of the National Insitutes of Health, but would hope that an agency such as the World Health Organization would also become involved in facilitating disussions leading to an enumeration of the problems and the means to deal with them. In this way, the evaluation of the biohazards and the adoption of methods to deal with them would be the concern and responsibility of members of the entire world-wide scientific community. This seems particularly important since such experimentation is likely to be considered and/or performed by scientists regardless of geographic location.

I also concur with your forth recommendation for an international meeting next year to present new data and further discuss the problems associated with the types of genetic engineering research as defined above. Nevertheless, I feel that such a meeting will not be highly productive of recommendations unless this topic is discussed and debated at other scientific meetings prior to that time and unless those scientists engaged or interested in this research and its ramifications begin to informally exchange views not only on the potential biohazards of this research but also on the means to effectively deal with these biohazards. In light of this belief, I shall close by mentioning what to me are important biohazards and suggest guidelines for dealing with them.

Possible Means for Dealing with Biohazards Associated with Genetic Engineering Research.

I have subdivided the treatment of contending with potential biohazards of genetic engineering research into six major categories as described below. As a general commentary, my own suggestions suffer from a limitation of knowledge and experience in certain areas of this research, especially as they relate to plant and animal viruses. I should also indicate that I think any

given experimental situation will necessitate rather special guidelines and that the procedures to contend with any given biohazard cannot be universally applied. In addition to potential federal and/or international guidelines that may be established that would need to be complied with by scientists desiring to conduct such research, it might be well that scientists at public and private research institutions as well as at commercial firms that are interested or now engaged in this line of research have the advice, counsel, and approval of individual or institutional biohazards committees much as the human use committees are used to approve and supervise human research. In the suggestions that follow, I have recommended measures that may well be more stringent than necessary. I believe, however, that it behooves us to take these extra precautions until and if it is shown that they are no longer essential.

a) Training of personnel who participate in genetic engineering research.

All persons engaged in this line of research including scientific, technical and support personnel should have appropriate training in conducting various biological, biochemical and containment procedures and should be well aware of all of the biohazards associated with the work and the means to contend with these biohazards. These individuals should not only familarize themselves with all of the written procedures, but should probably be certified as competent to be engaged in this research by an institutional biohazards committee.

b) Construction of recombinant DNA molecules. The cleavage of viral, plasmid and/or chromosomal DNAs with restriction endonucleases and their reassociation under favorable conditions to form recombinant DNA molecules should be done with great care. The left-over products of such reactions should be chemically, physically or enzymatically destroyed prior to disposal. Indeed, those investigators utilizing restriction endonucleases for elucidating the structure of DNA molecules should likewise take precautions to preclude introduction of such DNA fragments into the environment.

c) Introduction of recombinant DNA (Types 1, 2 and 3) or non-recombinant DNA (Type 4 experiments) molecules into other organisms.

i. Bacteria. Probably the most severe biohazards are encountered during the introdution of such DNA molecules into a bacterium, especially one like E. coli. In all aspects of this research, great care must be taken. It is, therefore suggested that these experiments be conducted in facilities that are designed for containment such as those that would be used for research with highly virulent viral or bacterial pathogens. This would include use of biohazard hoods, centrifuges equipped with containment hoods with ultrafiltration of exhaust air, etc. All of these items should be contained in a sealed room capable of being disinfected and with ultrafiltration of both incoming and exhaust air and double-door vapor-lock entrance and exit ports. Foot

washes and clothes changing facilities for personnel using the room might also be advisable. Since all material used in such experiments would require sterilization prior to disposal or dishwashing, a double-door autoclave should be mounted in the wall of such a containment room. Special facilities should also be provided for storage of the genetically altered microorganisms.

ii. Plant or animal cells in culture. The same precautions as outlined above should be utilized in experiments of this type if the DNA molecules introduced have the potential for autonomous replication and/or the possibility for being encapsulated in viral coats. It such is not the case, as would be true in some experiments attempting to genetically transform plant and animal cells, such precautions need not be taken since it is highly improbably that such cells could survive in the absence of the highly specialized culture media in which they grow and there would be little or no biohazard if such material were inadvertantly spilled or disposed of. Nevertheless, upon completion of such experiments the byproducts and/or materials left over should be disposed of by disinfection and/or autoclaving.

iii. Intact plants or animals. Here again, the precautions to be taken depend on whether the DNA molecules used have the potential for self-replication, transmissibility, and/or heritability. If so, the experiment should be done in isolators like those utilized in germ-free or gnotobiotic research and these isolators should be kept in containment rooms as described above. Similar requirements would be essential in instances in which bacteria harboring recombinant DNA molecules might be introduced into plants or animals. In other instances where there is absolutely no danger of pathogenesis due to the added DNA or heritability of the genetic information from one generation to the next, the experiments could be conducted with little more precaution than is taken in conventional experiments utilizing plants and animals.

d) Tests on products by genetically altered organisms. In most instances this will involve experiments with genetically altered microorganisms and the bacteria in question should be grown under the same conditions as were used in the experiments that produced them. The cells should be lysed or extracted and these extracts tested for sterility prior to taking the material into a general research laboratory for further processing. If a specific purified product is to be isolated and characterized, then the procedures used can be rather conventional. On the other hand, if the altered organism is to be utilized as a test vaccine then precautions need to be taken to protect the investigator from exposure and special cages should be utilized to house the animals being challenged with this antigen. If, perchance, the altered organism is a plant or animal species, then the precautions to be taken will depend on whether the organism contains a potentially infectious organism or agent or has a heritable trait. The appropriate guidelines established above should be adhered to in either case.

e) Tests on properties of altered organisms. Some experiments of this type will be necessary, especially if the purpose of the original experiments was to develop an organism to eventually be released into the ecosystem. It is thus rather difficult to suggest specific proposals for coping with the biohazards associated with this line of research. Clearly, experiments with altered microorganisms need to be conducted in facilities normally utilized for working with gnotobiotic animals with all of the precautions being taken as outlined above. Tests on animal and plant species are much more difficult to envision, however, and in either case would depend on the nature of the genetic alteration and whether it contributed to infectivity or was heritable.

f) Use of products and/or organisms. Licensing of the use of a product produced by a genetically altered organism or a preparation of the organism as a vaccine should adhere to the standards and requirements of licensing established by various federal agencies such as the Food and Drug Administration in the U.S.A. and equivalent licensing authorities in other countries. The introduction of a genetically altered organism into the ecosystem, however, should require conformance with requirements set forth by some international agency. In either case, data on efficacy and potential biohazards of the use of these products or organisms would need to be corroborated by more than one group of research scientists.

The above are just general guideline suggestions and it would seem worthwhile to me if individual scientists interested in this line of research would prepare detailed proposals pertaining to a specific type of experiment. In this way an accumulation of such proposals might better define the particular problems and the means to contend with them.

Concluding Remarks.

This memorandum would not be complete unless I responded in some way to the general concerns about academic freedom and regulation of science that your initial letter elicited. By way of illustrating my own feelings on these matters, I need only remind others that scientists working in the area of radiation research and atomic energy did not heed the early warnings of the potential hazards and many suffered material damage before central agencies were established to place regulations on the conduct of this research and to protect the general public from radiation hazards. In this case, it seems that the scientists most knowledgable about the dangers were altogether too slow in addressing themselves to the problems and in alleviating much of the pain and suffering which ensued. In the instances of regulation of human and/or animal research, again scientists were remiss in establishing guidelines for the conduct of their own experiments and because of the few who did not adhere to the high standards of the majority, restrictive regulations

were established that had to be adhered to by all. In the particular instance of research on genetic engineering it seems we have an opportunity to take a responsible stand that will establish the guidelines to permit this research to be conducted in a safe and beneficial way in the absence of unwarranted restrictions that would all but make this research impossible. Even though I disagree with you on some particulars I would thus like to take this opportunity to applaud you for your highly responsible and unselfish actions in initiating this dialog that will ultimately result in the safe resumption of this important area of research for the betterment of all.

Roy Curtiss III
Birmingham, Alabama, U.S.A.

RC/kb:blm